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Aurora A overexpression and pVHL reduced expression are correlated with a bad kidney cancer prognosis

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Abstract. We investigate the expression and localization of the tumor suppressor protein pVHL as well as the oncoprotein Aurora A kinase in kidney cancer. Both Aurora A kinase and pVHL protein status were evaluated using immunohistochemistry. The Aurora A expression is correlated with the Fuhrman grade and the TNM stage, while the pVHL expression is correlated with the capsule rupture and the TNM stage. Aurora A kinase expression increases in malignant tissue comparing to the non-malignant one. And there is a decrease in pVHL expression from the adjacent healthy tissues to the tumor's ones. The two kinds of opposite tumor profiles display significant distribution difference according to TNM stages. It could be proposed that the absence of Aurora A protein associated with a strong expression of pVHL in clear cells kidney carcinoma are of good prognosis for the disease.

Keywords: pVHL, Aurora A, kidney cancer, immunohistochemistry

1. Introduction

Renal cell carcinoma (RCC) represents 2–3% of all adults' malignant diseases. It is the most lethal form of urological cancer: local signs and symptoms when occurring appear at an advanced stage of the tumor and have negative prognostic implications [1]. Generally this disease is asymptomatic and 74% of renal tumors are discovered incidentally during an ultrasound or an abdominal scan requested for something else.

This cancer has a poor prognosis with a rate of 5 years survival around 15% [2], because the discovery is often

at late phase or even metastatic phase (20% to 30%) [3]. The high rate of mortality is due not only to the lack of early warning signs and symptoms, but also to the lack of effective treatments for patients at a late stage cancer. The affected people are often aged between 50 and 70 years old. This cancer is rare in children and young adults [4].

There are several histological subtypes of RCC including clear cell, papillary, chromophobe, and oncocytoma variants. But the clear cell Renal Cell Carcinoma (ccRCC) constitutes the most common and aggressive subtype [1]. Despite an increased detection of small kidney masses with cross-sectional imaging and subsequent removal with surgery, mortality has not decreased. So we need to find more tools to improve understanding and management of this disease. The abro-

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gation of the cell cycle mitotic spindle checkpoint has been involved in the progression of RCC [5,33].

Aurora-A is a crucial protein in cell division and many aberrations are assigned to its dysregulation such as genomic instability, abnormal duplication of the centrosomes and cell transformation. Besides, Aurora A could function as an oncogene through the abnormal regulation of centrosome function and this protein is designated as an essential actor in aneuploidy. Given its important role in the process of cell cycle, its overexpression in several cancers that affect: breast [6], colon [7], esophagus [8], ovary [9] has been reported. The expression of this protein was also studied in the kidney cancer [10–12]. Aurora A is a cell cycle proto-oncogenic serine/threonine kinase, which orchestrates the proper running of mitosis. It controls the cell entry into mitosis [13], the maturation and separation of centrosomes [14] and the assembly and stability of the mitotic spindle [13]. It participates also in cytokinesis [15]. This centrosomal protein plays a key role in regulating the cell cycle via its interaction with p53 protein, which is a transcription factor and a well-known tumor suppressor, thus, inducing cell cycle arrest or apoptosis of the cell when DNA is damaged [16,17].

Finally, at the end of mitosis Aurora A is ubiquitinated and therefore recognized by the 26S proteasome and degraded through the ubiquitin-proteasome pathway APC (Anaphase-Promoting Complex). Aurora A kinase is induced by inactivation of pVHL, a tumor suppressor protein [18].

Inactivation of the Von Hippel Lindau (VHL) tumor suppressor gene [19,20] is an early, causal event in the development of clear cell renal cell carcinomas and is common in both hereditary and nonhereditary forms. VHL is the main inactivated gene in ccRCC, with mutations detected in 40–80% of ccRCC patients [21–25].

The pVHL protein has several functions, the best documented of these functions relates to its role as the substrate-recognition component of multisubunit ubiquitin ligase complex that also contains elongin B, elongin C, cullin 2, and rbx1 [26]. This E3 ligase is involved in the ubiquitin proteasome pathway to degrade the endogenous proteins: the major proteolytic system in the eukaryotic cell. This tumor suppressor protein controls also the cell cycle exit [27,28] participates in the assembly and stability of the mitotic spindle [29, 30] fibronectin binding, and extracellular matrix assembly [26] and it is involved in the activity of centrosomes [31].

The pVHL protein plays a role in chromosome stability [27] and its absence increases aneuploidy. More-

over it's involved in the stabilization of the p53 protein, pVHL directly associates and positively regulates the tumor suppressor p53 by inhibiting Mdm2-mediated ubiquitination, Thus pVHL appears to play a crucial role in tumor suppression by participating actively as a component of p53 transactivation complex during DNA damage response, and therefore playing an important role in cell cycle regulation [27] and regulating the tumor growth and cytokinesis [32,33].

The protein pVHL is present in the cell under three isoforms as following: The isoform 1 contains exons 1, 2 and 3, it encloses 213 amino acid (AA), has as molecular weight of 30 kDa VHL30 [34] and resides mainly in the cytoplasm [30]; the isoform 2 contains only exon 1 and 3 corresponding to a protein with 173 AA and a molecular weight of about 24 kDa; The isoform 3 contains 161 AA with a size of 19 kD, it was formed as a result of internal translational initiation at an in-frame start codon (ATG) at codon 54 [35]. VHL19 resides primarily in the nucleus [30].

The expression status of tumor suppressor pVHL has been studied in several cancers including ovarian and uterine cancer ones [45], the colorectal cancer and liver metastases [36], as well as in the chondrosarcoma [37]. The expression of this protein was also studied in the kidney [38–41].

Recent studies showed a specific interaction between the oncoprotein Aurora A and the tumor suppressor protein pVHL. These studies included three parts and started with a study in bioinformatics where the interaction '*in silico*' was predicted with strong probability, and then an '*in vitro*' test using a recombinant protein was done and confirmed this interaction. Finally an '*in vivo*' study with endogenous proteins on cell cultures comes to consolidate this hypothesis [47,48].

In the present paper, we investigate the expression of Aurora A kinase and pVHL protein in kidney tumors of CCRCC subtype according to clinical and anatomopathological statuses.

2. Material and methods

2.1. Clinical tissue sample

79 archival paraffin-embedded tumor sections were collected from the Pathology department in Charles Nicolle Hospital-Tunis. They were obtained from patients who underwent surgery with pathologically confirmed kidney cancer: clear renal cell carcinoma, admitted to the Department of Urology between 2001 and

Table 1
Patient characteristics ($n = 79$)

Characteristic	Number of patients (%)
Age (years)	
Median	60
Range	36–87
Sexe	
Male	42
Female	37
Tumor Size	
≤ 70 MM	44
> 70 MM	34
Unknown	1
Partial or complet nephrectomy	
Partial	9
Complete	69
Unknown	1
Right or left kidney	
Right	32
Left	43
Unknown	4
Renal capsule rupture	
Yes	20
No	57
Unknown	2
Fuhrman Grade	
I	27
II	35
III	14
IV	3
TNM stage	
T1	37
T2	20
T3	20
Unknown	2

2010. The following clinical data were collected: age, gender, histological subtype, Fuhrman grade, tumor size, status of the renal capsule and the TNM classification stage. The patients' characteristics are shown in Table 1. The study protocol was approved by the hospital head of the histopathology department.

2.2. Antibodies

For Aurora A protein detection we used a mouse monoclonal antibody (clone 35c1, Sigma) which was developed against the non-catalytic N-terminal domain of the kinase. This clone was tested at the UMR6061 in the Institute of Genetics and Development of Rennes, France [42]. For pVHL protein revealing we used a mouse monoclonal anti-Von Hippel Lindau protein (3F391/Ig33) from Novus Biologicals, Inc.

2.3. Immunohistochemical staining

Sections of thick $3 \mu\text{m}$ were prepared for each case and stained with Hematoxylin eosin (H&E) intended for histological examination.

We proceeded to the review of slides and to their classification before we begin our experimentation on tumors initially classified as clear cell carcinomas according to ancient criteria. In fact new identities: renal carcinoma associated with a translocation MITF/TFE, clear cell papillary carcinoma, and carcinoma associated with polycystic disease have been excluded from this study [43].

Briefly, we deparaffinized sections in xylene and we rehydrated them through graded alcohol, then we used a Novolink polymer detection system (RE7150-K, Leica, TN) as recommended in the instruction manual. The slides were incubated with the primary antibody against Aurora A at 1:50 or pVHL at 1:100 overnight in a humidity tray. On the one hand, a positive control was included in each batch: for Aurora A antibody, a slid of colorectal carcinoma known to overexpress the autologous antigen and for pVHL antibody, a slid of healthy kidney which normally expresses this protein were used (Figs 1A, C). On the other hand, a negative control was performed by omitting the primary antibody and as expected, it did not produce any visible background staining (Figs 1B, D).

The evaluation used for intensity of staining and sub-cellular localization was performed in a blinded manner. The intensity of staining was scored as absent (= 0), weak (= 1), moderate (= 2), or strong (= 3) (Figs 2 and 3).

2.4. Statistical analysis

The analysis of the results involving the expression of both proteins together with the various histological and clinical parameters were realized, using the chi-square performed with the program Statistical Package for Social Sciences (SPSS 13.0). We considered that results were statistically significant when p values were < 0.05 , asterisks (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

3. Results

In the 79 studied samples for Aurora A in tumoral tissue, 35 contain a non tumoral one. Only 36 malignant

Table 2
Aurora A and pVHL intensity and location in different tissues

Intensity	Aurora A		pVHL	
	Non malignant tissue	Malignant tissue	Non malignant tissue	Malignant tissue
0	25	36	0	0
1	7	9	2	6
2	3	7	8	17
3	0	27	12	13
Location				
Cytoplasmic	3	36	20	26
Nuclear and cytoplasmic	7	4	2	10
Nuclear	0	3	0	0

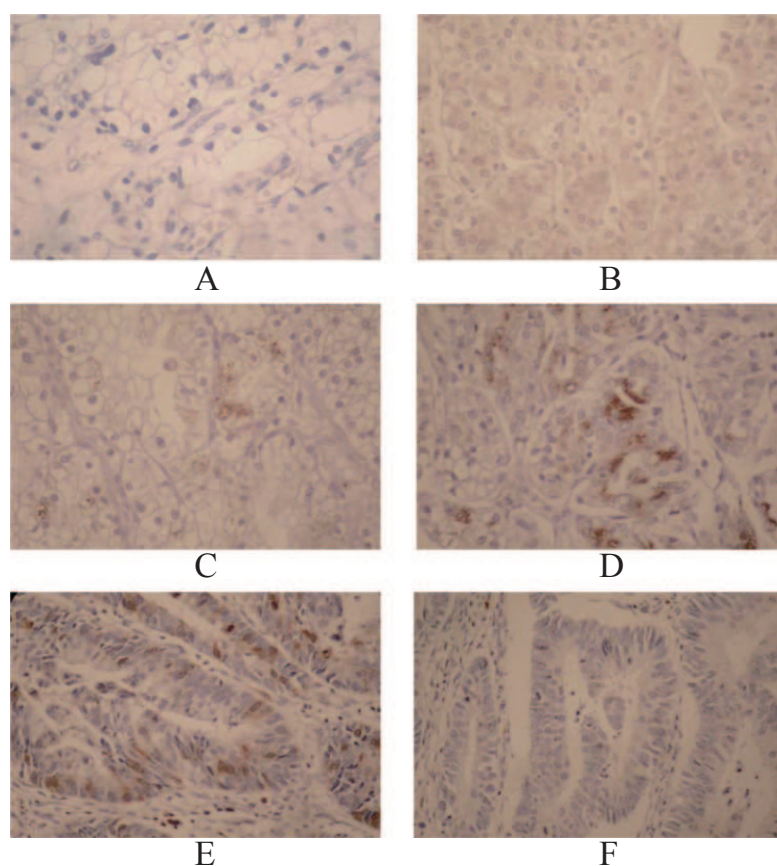


Fig. 1. Aurora A immunostaining in human tissue (x400). Brown color indicates antibody binding and intensity of staining. A, B, C and D: Immunostaining of malignant kidney tissue: (A): 0 negative staining, (B): + 1 weak positive staining, (C): + 2 moderate positive staining, (D): + 3 strong positive staining. E and F: Immunostaining of colorectal carcinoma (x400): (E): with anti-Aurora A antibody: positive control, (F): without anti-Aurora A antibody: négative control. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/DMA-2012-00942>)

tissues were evaluated for pVHL expression, 22 among them contain a non tumoral tissue.

Using the specific antibody to the Aurora A kinase, the expression of these proteins is investigated by immunohistochemistry on kidney tissue. Usually, Aurora A is absent in non- mitotic normal cells, its expression takes place only during mitosis. When it is expressed

in non-mitotic cells the oncoprotein is considered to be overexpressed. Accordingly, Aurora A is overexpressed in 43 (54.5%) of 79 kidney malignant tissues and in 10 (26.5%) of 35 non-malignant adjacent tissues. The modification of protein expression between the non-malignant tissue and the malignant one is supported by significant *p* value 0.01 (Table 2). This ex-

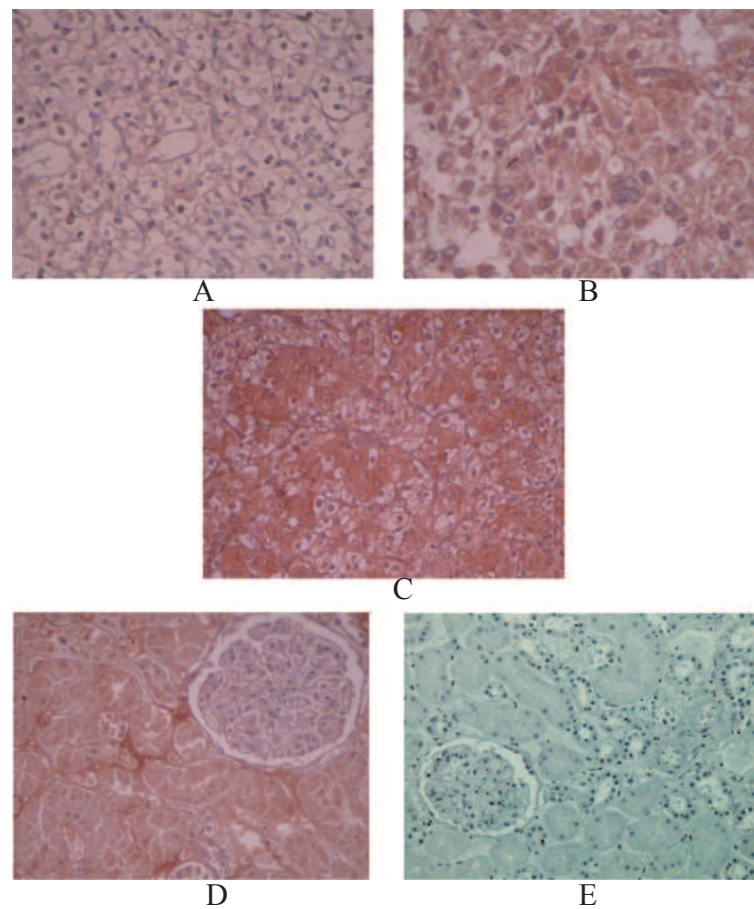


Fig. 2. pVHL protein immunostaining in human tissue (x400). Brown color indicates antibody binding and intensity of staining. A, B and C: Immunostaining of malignant kidney tissue (x400): (A): + 1 weak positive staining, (B): + 2 moderate positive staining, (C): + 3 strong positive staining. D and E: Immunostaining of healthy kidney tissue (x400): (D): with anti-pVHL antibody: positive control, (E): without anti-pVHL antibody: négative control. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/DMA-2012-00942>)

pression has been classified into four levels: 0, 1, 2 and 3; the level 0 corresponding to the absence of the protein expression, level 1 corresponding to weak expression, the level 2 corresponding to moderate expression and the level 3 corresponding to strong expression.

In non-malignant tissue, when Aurora A is overexpressed, it does not reach the level 3 of expression in the tissue. On the other side, 62.8% of malignant tissue expressing Aurora A display high intensity labeling 27/43 at level 3. This difference in high level expression between malignant and normal adjacent tissue is significant with $p = 0.0003^{**}$ (Table 2).

When Aurora-A is overexpressed we record the location of the protein. In malignant tissue Aurora A is 83.7% (36) cytoplasmic, 9.3% (4) both nuclear and cytoplasmic and 7% (3) with only nuclear localization. In adjacent non-malignant tissue the distribution of the Aurora A protein is as follows: 30% (3) cytoplasmic

and 70% (7) both nuclear and cytoplasmic. This difference in location between the non-tumor tissue and tumor one is supported by a significant p -value of 0.001^{**} (Table 2) with a great excess of cytoplasmic location in tumor tissue.

The expression of Aurora A kinase in tumor tissue is correlated with the Fuhrman grade with p value of 0.003^{*} (Table 3). The expression of Aurora A kinase in tumor tissue is also correlated with the TNM Classification of Malignant Tumours $p = 0.008^{**}$ (Table 3).

Using autologous antibody, the tumor suppressor protein pVHL expression is also investigated by IHC in 36 biopsies. In both neighboring healthy tissue and tumoral tissues, the pVHL is always expressed however with some differences in the level and location. In malignant kidney tissue 16.7% (6) display a weak intensity, 47.2% (17) intermediate intensity and 36.1% (13) show a high one. Contrariwise, in non-malignant

Table 3
Aurora A and pVHL expression with grade and stage

Aurora A intensity	Fuhrman grade				$p = 0.003$
	I	II	III	IV	
0	14	11	9	2	
1	5	1	2	1	
2	1	5	1	0	
3	7	18	2	0	

Aurora A intensity	TNM stage			$p = 0.008$
	T1	T2	T3	
0	15	12	8	
1	6	1	2	
2	1	0	6	
3	15	7	4	
Unknown	2			

pVHL intensity	Fuhrman grade				$p = 0.054$
	I	II	III	IV	
1	2	3	1	0	
2	6	7	1	3	
3	3	4	6	0	

pVHL intensity	TNM stage			$p = 0.026$
	T1	T2	T3	
1	2	1	3	
2	5	3	9	
3	5	6	1	
Unknown	1			

tissue distribution is as following: 9.1% (2) with low intensity, 36.4% (8) with moderate intensity and 54.5% (12) with strong intensity. The difference in protein expression between adjacent healthy and tumor kidney tissues is supported by a p value equal to 0.01 (Table 2).

The Von Hippel Lindau protein exhibits a difference in location between tumor tissue and healthy adjacent tissue. Indeed in the tumor tissue, this expression is often observed at a cytoplasmic level 72.2% (26 of 36), whereas in healthy adjacent tissue this expression is mainly 90.9% (20 of 22) cytoplasmic. The difference between the two tissues is not significant (Table 2). However, it seems that pVHL is also expressed at a nuclear level along with cytoplasmic location in 27% of tumor tissues whereas only 10% of healthy tissues display such expression profile.

The expression of pVHL in tumoral tissue according to the grade of Furman is described in Table 3. This expression seems to be similar for the different grades. The decrease of pVHL protein expression in tumor tissue is correlated with the renal capsule rupture (p value = 0.027*) as seen in Table 3. It's also correlated with the advanced T3 stage according to TNM Classification $p = 0.002^*$ (Table 3).

The expression of Aurora A and pVHL does not significantly vary according to age, sex and tumor size.

Considering both markers, the tumor suppressor protein pVHL strong expression associated to a weak ex-

pression of the oncoprotein Aurora A could be regarded as the best prognosis profile and is indeed shown to be the most frequent in T1 stage. On the contrary, the over expression of Aura A and pVHL decrease could be considered as bad prognosis criteria. According to TNM classification, we found that such tumor profiles are frequent in T3 stage. The two kinds of opposite tumor profiles display significant distribution difference according to stages with p value of 0.024*.

4. Discussion

In this study, evidence has been provided for an overexpression of the oncoprotein Aurora A kinase in tumoral kidney cancer tissue, and thus confirming the previous studies [10–12].

When it was available, we also interestingly noted an overexpression of the protein in normal adjacent tissue up to 26.5%, suggesting that this could be an early event in tumor genesis [11,44].

Our comparative study of the expression of this protein between tumor and healthy adjacent tissue pinpoint the change of expression in the progression from the healthy to tumor status and in agreement with the description of the kinase as being an oncoprotein.

The strong expression of the Aurora A protein level qualified by an expression equal to 3, is obvious in more than the half of the analyzed tumoral tissues, while in the healthy tissue such expression level is never reached; which is consistent with the known role of this protein.

Protein kinase Aurora A expression is predominantly cytoplasmic, which is in agreement with previous studies [6–8,44]. While in normal tissue, such expression was mainly both nuclear and cytoplasmic. This difference in protein location needs to be better investigated and explained according to its functions and to its altered structure and/or dysregulation in tumor cells.

The pVHL protein expression was positive in 100% of the studied tissues: tumor as well as adjacent healthy tissue, which is in accordance with the studies done [40, 41,45]. An apparent decrease was recorded in the expression level of the protein pVHL from healthy to malignant tissues which is in agreement with the main function of the protein as a tumor suppressor and with a previous study of Horng 2003 [40].

Lin 2008, Horng 2003 and Corless 1997 [40,41,45] found that the expression of the pVHL protein is cytoplasmic in 100% of the studied tissues, while in our work, the staining was chiefly cytoplasmic in addition

to mixed nuclear and cytoplasmic staining. The antibody used in our work, recognizes isoform1 (major form) and isoform2 of pVHL. The major form is both cytoplasmic and nuclear [46]. In their respective work, Lin 2008, Horng 2003 and Corless 1997 [40,41,45] used antibodies expecting to bind all isoforms and have recorded only the cytoplasmic presence of the protein.

Our work is in agreement with that of scharmal 2003 [39], and brings to light, for the first time, a link between pVHL protein expression and the grade of Furhman, TNM classification and the renal capsule rupture, for which statistically significant associations exist.

We suggest that the pVHL protein strong expression protects against high grade and stage, but since we have no indication about endogenous VHL gene mutations nor concerning their effects on VHL protein stability, this remains a mere supposition. There were a statistically significant association between Aurora A protein overexpression with the high grade of Furhman [11] and high stage TNM classification.

Indeed the pVHL protein staining is uniform while the Aurora A protein's is not. In healthy tissue, the pVHL protein expression is ubiquitous, whereas the Aurora A expression takes place only when the cell is in division, which may partly explain the heterogeneity of expression in malignant tissue. Altogether, the expression levels of the oncoprotein Aurora A and tumor suppressor pVHL correlate in an opposite manner and one has to better investigate to which extent a high level of Aurora A expression associated to a reduction in pVHL expression could be considered as a molecular signature of a high TNM stage and of a bad prognosis. Considering, Aurora A /pVHL interaction [47,48], it could be assumed that the decrease of pVHL could lead or at least participate to Aurora A overexpression [18]. Based on such results and on the observations of the present paper, it could be proposed that absence of Aurora A protein associated to a strong expression of pVHL in clear cells kidney carcinoma are of good prognosis for the disease.

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